BIOCHEMICAL CHARACTERIZATION OF HUMAN AND YEAST CHOLINE AND ETHANOLAMINE KINASES

by

SEE TOO WEI CUN

Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

April 2006

ACKNOWLEDGMENTS

I would like to thank my field supervisor, Dr. Manfred Konrad for his scientific guidance and his trust in me by giving me the independence to fulfill my curiosity in science. I am also very indebted to his enormous help during the preparation of this thesis and his kind hospitality, which has really enhanced the joy of my stay in Germany.

I would also like to express my sincere appreciation to Associate Professor Dr. Tengku Sifzizul bin Tengku Muhammad for his willingness to act as my formal supervisor and his invaluable help on many occasions. I am also grateful to Professor Dieter Gallwitz for giving me the opportunity to work in his department and Professor Zainul Fadziruddin Zainuddin, Professor Norazmi Mohd. Nor and Associate Professor Dr. Nazalan Najimudin for their advice and assistance.

I would like to acknowledge the contributions of Dr. Enrico Malito, Nikolina Sekulic and Associate Professor Dr. Arnon Lavie in solving the structure of human choline kinase. I thank Dr. Henning Urlaub and Dr. Jens Grüber for their help in protein mass spectrometry and RNAi experiments.

I am very thankful to Universiti Sains Malaysia for its generous financial support under the Academic Staff Training Scheme and staffs from Institute for Postgraduate Studies for their help in all aspects of my Ph.D candidature.

A very big ‘‘Thank you’’ to all my former collegues in Germany, especially to Stephan, Heike, Ursula, and Yujie for their friendships that made the three years of my life in Göttingen so special. Last but not least, I thank my wife Ling Ling, and my family for their love and support.

This thesis is dedicated to my wife Ling Ling,

for her patience and unwavering support,

and our son Zhi Xiang,

my source of inspiration and fascination.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

APS	Ammonium persulphate
bp	Base pair
BSA	Bovine serum albumin
C-	Carboxy-
cDNA	Complementary DNA
CK	Choline kinase
CL	Cardiolipin
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EK	Ethanolamine kinase
FCS	Fetal calf serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GST	Glutathione sulfur transferase
HC-3	Hemicholinium-3
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
IgG	Immunoglobulin G
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	Kilo Dalton
LB	Luria-Bertani
LDH	Lactate dehydrogenase

MBP	Maltose binding protein
MCS	Multiple cloning site
mRNA	Messenger RNA
M_w	Relative molecular weight
N-	Amino-
NADH	Nicotinamide adenine dinucleotide
OD	Optical density
PA	Phosphatidic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PCho	Phosphocholine
PE	Phosphatidylethanolamine
PEtn	Phosphoethanolamine
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PK	Pyruvate kinase
PMSF	Phenylmethylsulfonyl fluoride
PS	Phosphatidylserine
RNA	Ribonucleic acid
RT-PCR	Reverse transcription-polymerase chain reaction
SDS	Sodium dodecyl sulphate
siRNA	Small interference RNA
SM	Sphingomyelin
TAE	Tris-acetate-EDTA
TEMED	N,N,N',N'-tetramethyl-ethylenediamine

T_m	Melting temperature
Tris	Tris(hydroxymethyl)-aminomethane
U	Unit
v/v	Volume to volume
w/v	Weight to volume
x g	-fold gravity

Amino acids, nucleotides and deoxynucleotides are abbreviated according to the International Union of Pure and Applied Chemistry (IUPAC).

PENCIRIAN BIOKIMIA KOLINA KINASE DAN ETANOLAMINA KINASE DARIPADA MANUSIA DAN YIS

ABSTRAK

Kolina kinase (CK) dan etanolamina kinase (EK) merupakan enzim pertama di dalam biosintesis fosfatidilkolina dan fosfatidiletanolamina. Peningkatan aktiviti CK telah dikaitkan dengan tumor manusia. Oleh itu, perencatan CK telah dicadangkan sebagai strategi antikanser yang berpotensi. EK pula dicadangkan penting di dalam karsinogenesis dengan menggalakkan pertumbuhan sel. Kajian ini melaporkan pengklonan gen CK/EK dan pencirian biokimia kesemua isoform CK/EK manusia. Sejumlah enam gen CK/EK manusia (hCKα1, hCKα2, hCKβ, hEK1, hEK2α dan hEK2β) telah diklon daripada perpustakaan cDNA hati dan telah diekspreskan di dalam *Escherichia coli*. CK ditunjukkan berupaya memfosforilasi kolina dan etanolamina tetapi EK adalah spesifik terhadap etanolamina. Perbezaan panjang yang hanya 18 amino asid di antara hCKα1 dan α2 telah menyebabkan aktiviti isoform α1 50% lebih rendah. Yang menariknya, isoform hEK2β didapati tidak menunjukkan sebarang aktiviti enzim. Selain daripada enzim manusia, empat gen CK/EK daripada yis (yCK dan yEK daripada *Saccharomyces cerevisiae*, SpCK daripada *Schizosaccharomyces pombe*, dan AgCK daripada *Ashbya gossypii*) juga telah diklon, diekspreskan dan diciri secara biokimia. Tidak seperti homolog manusia, yEK didapati tidak spesifik terhadap etanolamina manakala AgCK pula mempunyai kespesifikan yang tinggi terhadap kolina. Kajian perencatan terhadap hCK/EK dan yCK mendedahkan bahawa hemikolinium-3 (HC-3) hanya efektif di dalam perencatan aktiviti isoform hCKα2 dan yCK bukan merupakan enzim model yang baik untuk digunakan bagi menguji keberkesanan perencat-perencat hCK. Kajian mutagenesis telah mengenalpasti satu residu aspartat yang kritikal untuk tindakbalas pemangkinan pada CK/EK manusia dan

yis. Residu penting untuk pemilihan kolina atau etanolamina sebagai substrat juga telah dikenalpasti. Eksperiman pemfosforilan menunjukkan bahawa hCKβ dan hEK2α boleh difosforilasikan oleh PKA dan PKC secara *in vitro*. Fosforilasi ini menyebabkan peningkatan sebanyak 1.5 dan 2.7 kali ganda aktiviti hCKβ dan hEK2α. Kajian ini juga telah menunjukkan penggunaan teknik gangguan RNA untuk menyelidik peranan hCKα dan hCKβ di dalam sel HeLa. ''Knockdown'' tunggal hCKα menyebabkan fenotip kematian manakala ''knockdown'' tunggal hCKβ tidak menunjukkan sebarang kesan jelas terhadap sel. Yang paling menarik, ''knockdown'' serentak kedua-dua isoform tersebut membolehkan sel tumbuh secara normal. Pemerhatian ini mencadangkan bahawa interaksi di antara hCKα dan β adalah penting untuk kemandirian sel.

BIOCHEMICAL CHARACTERIZATION OF HUMAN AND YEAST CHOLINE AND ETHANOLAMINE KINASES

ABSTRACT

Choline kinase (CK) and ethanolamine kinase (EK) are the first enzymes in the biosynthesis of phosphatidylcholine and phosphatidylethanolamine. Increased activity of CK has been implicated in human tumors. CK inhibition has therefore been proposed as a potential anticancer strategy. Likewise, EK is suggested to be important in carcinogenesis by promoting cell growth. This study reports the cloning of CK/EK cDNAs and biochemical characterization of the full set of human CK/EK isoforms. A total of six human CK/EKs homologs (hCKα1, hCKα2, hCKβ, hEK1, hEK2α and hEK2β) were cloned from liver cDNA library and expressed in *Escherichia coli*. CKs were shown to phosphorylate choline and ethanolamine, but EKs were specific for ethanolamine. The length difference of only 18 amino acids between hCKα1 and α2 resulted in more than 50% lower activity in the α1 isoform. Intriguingly, the hEK2β isoform did not show any detectable enzyme activity. Besides the human enzymes, four CK/EK genes from three yeast species (yCK and yEK from *Saccharomyces cerevisiae*, SpCK from *Schizosaccharomyces pombe*, and AgCK from *Ashbya gossypii*) were also cloned, overexpressed and their products were biochemically characterized. Unlike their human homologs, yEK was not specific for ethanolamine while AgCK was highly specific for choline. Inhibition studies on hCK/EKs and yCK revealed that hemicholinium-3 (HC-3) was only effective in inhibiting the activity of the hCKα2 isoform and that yCK was not a good model enzyme for testing the efficacy of hCK inhibitors. Mutagenesis studies have identified a single aspartate residue that was critical for catalysis in human and yeast CK/EKs. Important residues for substrate preference towards either choline or ethanolamine were also identified.

Phosphorylation experiments showed that hCKβ and hEK2α could be phosphorylated by PKA and PKC *in vitro*. The phosphorylations resulted in 1.5 and 2.7 fold stimulation of hCKβ and hEK2α activities. This work also demonstrated the use of the RNA interference technique to investigate the role of hCKα and β in HeLa cells. Single knockdown of hCKα resulted in lethal phenotype while single knockdown of hCKβ did not show any apparent effect on the cells. Remarkably, the simultaneous knockdown of both isoforms resulted in normal cell growth. These observations suggested that the interaction between hCKα and β was important for cell survival.

CHAPTER 1.0 INTRODUCTION

1.1 Phospholipids: Structures and functions

Phospholipids are generally divided into two groups: Phosphoglycerides (also known as glycerophospholipids or glycerophosphatides) and sphingophospholipids (also known as sphingomyelins). Phospholipids generally refer to phosphoglycerides since sphingophospholipids are usually classified as the lipid class of sphingolipids (McKee & McKee, 2003). Despite their structural differences all phospholipids have hydrophobic and hydrophilic domains. The hydrophobic domain is composed largely of the hydrocarbon chains of fatty acids while the hydrophilic domain that consists of the polar head group, contains phosphate and other charged or polar groups (McKee & McKee, 2003).

All phosphoglycerides are derived from *sn*-Glycerol-3-phosphate (Figure 1.1). Esterification of this alcohol with two fatty acids at positions C-1 and C-2 gives a phosphatidate. Formation of phosphate diester linkage at position C-3 to certain alcohols results in different phosphoglycerides as shown in Table 1.1. The phosphoglycerides are named and classified according to the nature of the alcohol esterifying the glycerol phosphate. In most phosphoglycerides, the fatty acid substituted on C-1 is saturated and that on C-2 is unsaturated (Kuchel & Ralston, 1998). It must be noted that about 1% of the total phosphoglycerides that occur in animal cells are in the form of lysophosphoglycerides, in which one of the acyl substituents, usually from C-2, is missing (Kuchel & Ralston, 1998). The lysophosphoglycerides are named by adding the prefix lyso- to the name of the parent phosphoglyceride.

Phospholipids have the most important function in living organisms by forming major structural components of membranes. Table 1.2 shows the lipid compositions of

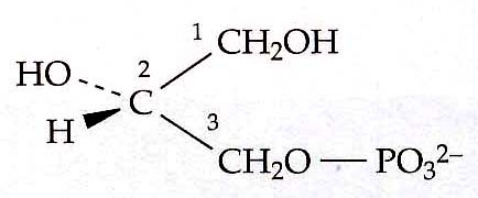
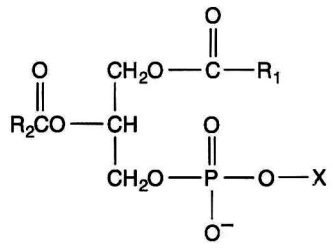


Figure 1.1: Structure of *sn*-Glycerol-3-phosphate. Numbers indicate carbon positions (Metzler & Metzler, 2001).

Table 1.1: Major classes of phosphoglycerides. R_1 and R_2 represent the two ester linked fatty acid chains. Table adapted from McKee & McKee (2003).



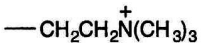
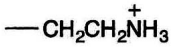
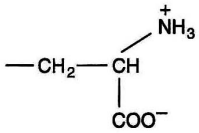
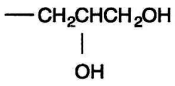
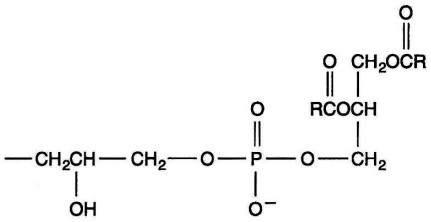
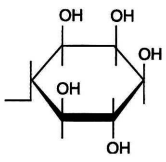
X Substituent		
Name of X-OH	**Formula of X**	**Name of Phospholipid**
Water		Phosphatidic acid (PA)
Choline		Phosphatidylcholine (PC)
Ethanolamine		Phosphatidylethanolamine (PE)
Serine		Phosphatidylserine (PS)
Glycerol		Phosphatidylglycerol (PG)
Phosphatidylglycerol		Diphosphatidylglycerol or cardiolipin (CL)
Inositol		Phosphatidylinositol (PI)

Table 1.2: Phospholipid composition of membranes. PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PI, Phosphatidylinositol; PS, Phosphatidylserine; PG, Phosphatidylglycerol; CL, Cardiolipin (Diphosphatidylglycerol); SM, Sphingomyelin. Sources: Adapted from Metzler & Metzler (2001) and Zubay (1998).

	Phospholipid composition (% of total lipids)						
Source	PC	PE	PI	PS	PG	CL	SM
Rat liver							
Plasma membrane	18	11	4	9	-	-	14
Rough endoplasmic reticulum	55	16	8	3	-	-	3
Inner mitochondrial membrane	45	25	6	1	2	18	3
Nuclear membrane	55	20	7	3	-	-	3
Golgi	40	15	6	4	-	-	10
Lysosomes	25	13	7	-	-	5	24
Rat brain myelin	11	14	-	7	-	-	6
Rat erythrocyte	31	15	2	7	-	-	9
Human erythrocyte	17	16	1	1	-	-	16
***E. coli* plasma membrane**	0	80	-	-	15	5	-

different membranes and organisms. Phosphoglycerides are the most abundant phospholipids found in the cell membrane (McKee & McKee, 2003). Several phospholipids also act as emulsifying agents and surface active agents (Kuroki & Voelker, 1994). Phospholipids are suited to these roles because of their amphipathic properties (Metzler & Metzler, 2001). Interest in phospholipid metabolism has also blossomed in the past 15 years or so with the discovery of phospholipids being involved in key regulatory functions in mammalian cells (Vance & Vance, 2004). In the nucleus, phospholipids are important components of chromatin and they also serve as a pool for intranuclear signaling molecules (Albi & Viola Magni, 2004).

1.1.1 Phosphatidic acid

Phosphatidic acid (PA) is the simplest phosphoglyceride and is not an abundant lipid constituent in living organisms. The most important role of phosphatidic acid is acting as precursor for the synthesis of all other phosphoglycerides (Zubay, 1998). Dephosphorylation of phosphatidic acid by phosphatidate phosphatase yields diacylglycerol, which is a precursor for the synthesis of phosphatidylcholine and phosphatidylethanolamine via the Kennedy pathway. Diacylglycerol can also be further acylated to form triacylglycerol (Athenstaedt & Daum, 1999). It is also widely accepted that the primary role of diacylglycerol is to activate protein kinase C (PKC) which phosphorylates a range of cellular proteins (Nishizuka, 1986).

1.1.2 Phosphatidylcholine

Phosphatidylcholine (PC) is also known as lecithin. It is the most abundant phospholipid in animal and plants, often amounting to almost 50% of the total phospholipid content as it is the key building block of membrane bilayers (Billah & Anthes, 1990). Although PC is considered to be less characteristic of bacterial cell membranes, a recent estimate suggests that probably more than 10% of all bacteria contain PC as a membrane phospholipid (Sohlenkamp *et al*., 2003). In mammals, PC

is also the principal phospholipid in bile, lung surfactant, and plasma lipoproteins, and plays a critical role as second messenger in signal transduction (Vance, 2002). PC serves as a phospholipid pool for signaling molecules such as diacylglycerol and phosphatidic acid, which are produced from the hydrolysis of PC by phospholipase C and phospholipase D, respectively (Billah & Anthes, 1990).

Perturbation of PC homeostasis has been implicated in cell death either by necrosis or apoptosis (Cui & Houweling, 2002). Evidence that reduction of PC synthesis alone can trigger apoptosis was first found in a cell line having a temperature-sensitive defect in phosphocholine cytidyltransferase, the second enzyme in PC synthesis via the CDP-choline pathway (Cui *et al*., 1996). Edelfosine, an antitumor PC analog, has been demonstrated to inhibit PC synthesis, and this correlated with inhibition of cell growth in various cancer cell lines (Wright *et al*., 2004).

PC is the major storage form for choline inside the brain. Choline acetyltransferase synthesizes the cholinergic neurotransmitter acetylcholine, from choline (Lee *et al*., 1993). Coupling between phosphatidylcholine hydrolysis and acetylcholine synthesis in the brain has been proposed (Blusztajn *et al*., 1987).

1.1.3 Phosphatidylethanolamine

Phosphatidylethanolamine (PE) is the major phospholipid in bacterial membranes and the second most abundant phospholipid in eukaryotes (Mazzella *et al*., 2004). PE is also known as cephalin (McKee & McKee, 2003). Unlike other eukaryotic cell membranes, *Drosophila*'s major phospholipid is PE instead of PC, with PE comprising about 55% of the total membrane phospholipids (Jones *et al*., 1992). In liver and yeast, PE serves as precursor for the synthesis of PC by phosphatidylethanolamine N-methyltransferase (PEMT) pathway (Carman & Henry, 1999; Noga & Vance, 2003). PE is also involved in the direct post-translational modification of proteins to allow their

association with membranes. Thus, it was shown that the yeast protein Apg8p, an essential factor for autophagy, is reversibly modified with PE (Kirisako *et al*., 2000). The amount of PE within the lipid bilayer determines membrane structure and fluidity (Yeagle, 1989).

1.1.4 Phosphatidylserine

Phosphatidylserine (PS) is the only amino acid containing phosphoglyceride. Although it is widely distributed in animals, plants and microorganisms, it usually represents less than 10% of the total phospholipids. PS is located entirely on the inner monolayer surface of the plasma membrane and other cellular membranes (Manno *et al*., 2002). This distribution is disturbed during platelet activation and cellular apoptosis (Martinez *et al*., 2006). PS is known to externalize to the cell surface in apoptotic cells and believed to be important for the recognition and removal of apoptotic cells by phagocytes (Yoshida *et al*., 2005); it also serves as important cofactor for the activation of protein kinase C and other enzymes such as Na^+/K^+ ATPase and neutral sphingomyelinase, and is involved in the blood coagulation process in platelets (Lentz, 2003).

1.1.5 Phosphatidylglycerol

Phosphatidylglycerol (PG) is the main component of some bacterial membranes, for example, *E. coli* has up to 20% of PG in its membrane (Mazzella *et al*., 2004). In plants, PG is required for photosynthetic function in the thylakoid membrane (Sato *et al*., 2000); it is especially important as a component of the lung surfactant in animals (Hallman & Gluck, 1976). Moreover, PG has been reported to function as precursor for the biosynthesis of the poly(glycerol phosphate) backbone of lipoteichoic acid in bifidobacteria (Op den Camp *et al*., 1985).

1.1.6 Diphosphatidylglycerol

Diphosphatidylglycerol, also known as cardiolipin (CL), is found almost exclusively in energy transducing membranes such as the bacterial cytoplasmic membrane and the inner membrane of mitochondria (Wright *et al*., 2004). The content of CL in the membrane becomes a critical factor for mitochondrial function under different stress conditions such as temperature changes, low substrate level and oxidative stress (Schlame *et al*., 2000). Interaction of CL with a large number of mitochondrial proteins can lead to functional activation of certain enzymes, especially those involved in oxidative phosphorylation (Schlame *et al*., 2000). CL may also have a specific role in the import of proteins into mitochondria and regulation of gene expression (Wright *et al*., 2004).

CL is thought to play a decisive role in apoptosis by the recruitment of cytosolic proteins to mitochondria and membrane permeabilization for the concomitant release of apoptogenic mitochondrial proteins (Wright *et al*., 2004). Thus, it has been demonstrated to be tightly associated with cytochrome c. Since release of cytochrome c into the cytoplasm is a major regulatory event during the induction of apoptosis, this is a rate limiting step in the formation of apoptosome for activation of executioner caspases (Wright *et al*., 2004).

1.1.7 Inositol-containing phospholipids

Inositol-containing phospholipids are generally known as phosphoinositides; they constitute 2 to 8 % of the lipids in eukaryotic cell membranes but are metabolized more rapidly than other lipids (Majerus *et al*., 1988). Animal cells contain three distinct species of inositol-containing phospholipids, the most abundant being phosphatidylinositol (PI). PI can be sequentially phosphorylated by specific kinases leading to the production of phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-biphosphate (PIP_2), by addition of phosphate groups to

hydroxyl residues in the inositol ring as shown in Figure 1.2 (Campbell & Smith, 2000). The release of inositol-1,4,5-triphosphate and diacylglycerol from PIP_2 is catalyzed by phospholipase C (Figure 1.2). Inositol-1,4,5-triphosphate is a signaling molecule that promotes the release of intracellular calcium, and diacyglycerol is a signaling molecule that activates several isoforms of protein kinase C (Berridge & Irvine, 1984).

1.1.8 Sphingophospholipids

Sphingophospholipids, also known as sphingomyelin (SM), are primary phospholipids in brain (10% of total phospholipid) and erythrocytes of ruminant animals, but it does not appear to occur in plants or microorganisms (Zubay, 1998). In a single cell, SM is present in higher percentage in the nucleus compared to other phospholipids and its amount changes in different cell physiological states (Albi & Viola Magni, 2004). SM is hypothesized to play a role in RNA maturation by protecting part of the RNA from enzyme digestion prior to exportation from the nucleus (Albi & Viola Magni, 2004). SM may also act to stabilize DNA as a decrease in SM concentration during S-phase was reported to favor the unwinding of DNA (Albi *et al*., 1997; Albi & Viola Magni, 2004). SM and cholesterol may be located together in specific sub-domains of membranes to form lipid rafts, which have been associated with cellular signalling, virus trafficking, intracellular transport, and budding of yeasts (van Meer & Lisman, 2002). The importance of SM in signal transduction is also linked to the action of sphingomyelinase on SM to form ceramide, a widely known second messenger for many pathways including apoptosis (Pettus *et al*., 2002).

1.2 Biosynthesis of major eukaryotic membrane phospholipids: Phosphatidic acid, phosphatidylethanolamine, phosphatidylcholine and phosphatidylserine

Eukaryotic cell membranes contain more than a thousand different phospholipid species as a result of the diverse mixture of acyl chains at the sn-1 and sn-2 positions and the variety of polar head groups (Vance & Vance, 2004). Studies of eukaryotic

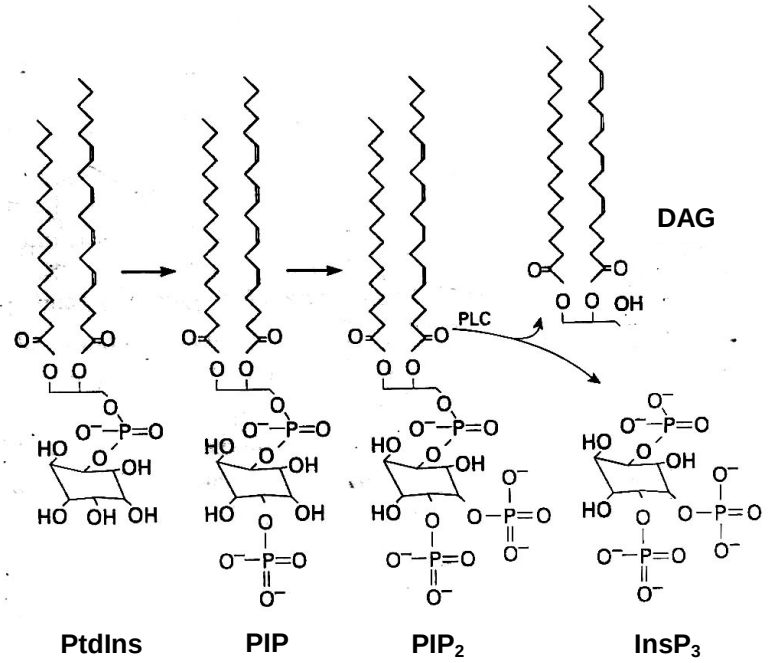


Figure 1.2: Phosphorylation and breakdown of phosphatidylinositols. Phosphatidylinositol (PtdIns) can be successively phosphorylated on positions 4 and 5 of the inositol ring to give phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-biphosphate (PIP_2). The release of diacylglycerol (DAG) and phosphorylated inositol derivatives, such as inositol 1,4,5-triphosphate ($InsP_3$) that function in cell signaling, is catalyzed by specific phospholipase C (PLC). Figure adapted from Campbell & Smith (2000).

phospholipid biosynthesis have a long history, dating back to the 1950s when most of the pathways were delineated, mostly by Eugene Kennedy's work (Kent, 1995). Identification of the genes and gene products involved in the biosynthesis of major phospholipids were slow due to difficulties encountered in purifying the respective enzymes as many of them are membrane-associated (Kent, 1995; Vance & Vance, 2004). Nevertheless, most of the genes involved in the biosynthetic pathways of phospholipids have been identified.

1.2.1 Synthesis of the backbone, phosphatidic acid

For the synthesis of PA, glycerol-3-phosphate is first converted into 1-acylglycerol-3-phosphate (lysophosphatidate) by glycerol-3-phosphate acyl-transferase (Figure 1.3). Two isoforms of this enzyme, which are encoded by two separate genes and have distinct subcellular localization have been identified (Vance & Vance, 2004). The second acylation step is catalyzed by acyltransferase, the donor of fatty acyl chains of phosphatidate is acyl-CoA. Five different acyl-CoA synthetases have been identified, they differ in their subcellular localization and acyl chain preference (Vance & Vance, 2004).

PA can be hydrolyzed by phosphatidate phosphatase to yield diacylglycerol, or converted to CDP-diacylglycerol by CDP-diacylglycerol synthase (Figure 1.3). Diacyglycerol is required for the synthesis of PC, PE and PS. CDP-diacylglycerol is used for the synthesis of PI, PG and CL. Cloning of mouse and human phosphatidate phosphatase cDNAs have been reported (Kai *et al*., 1996; Sun *et al*., 2005). Two genes encoding human CDP-diacylglycerol synthase have also been reported and the two isoforms are believed to have different cellular localization according to their functions (Vance & Vance, 2004).

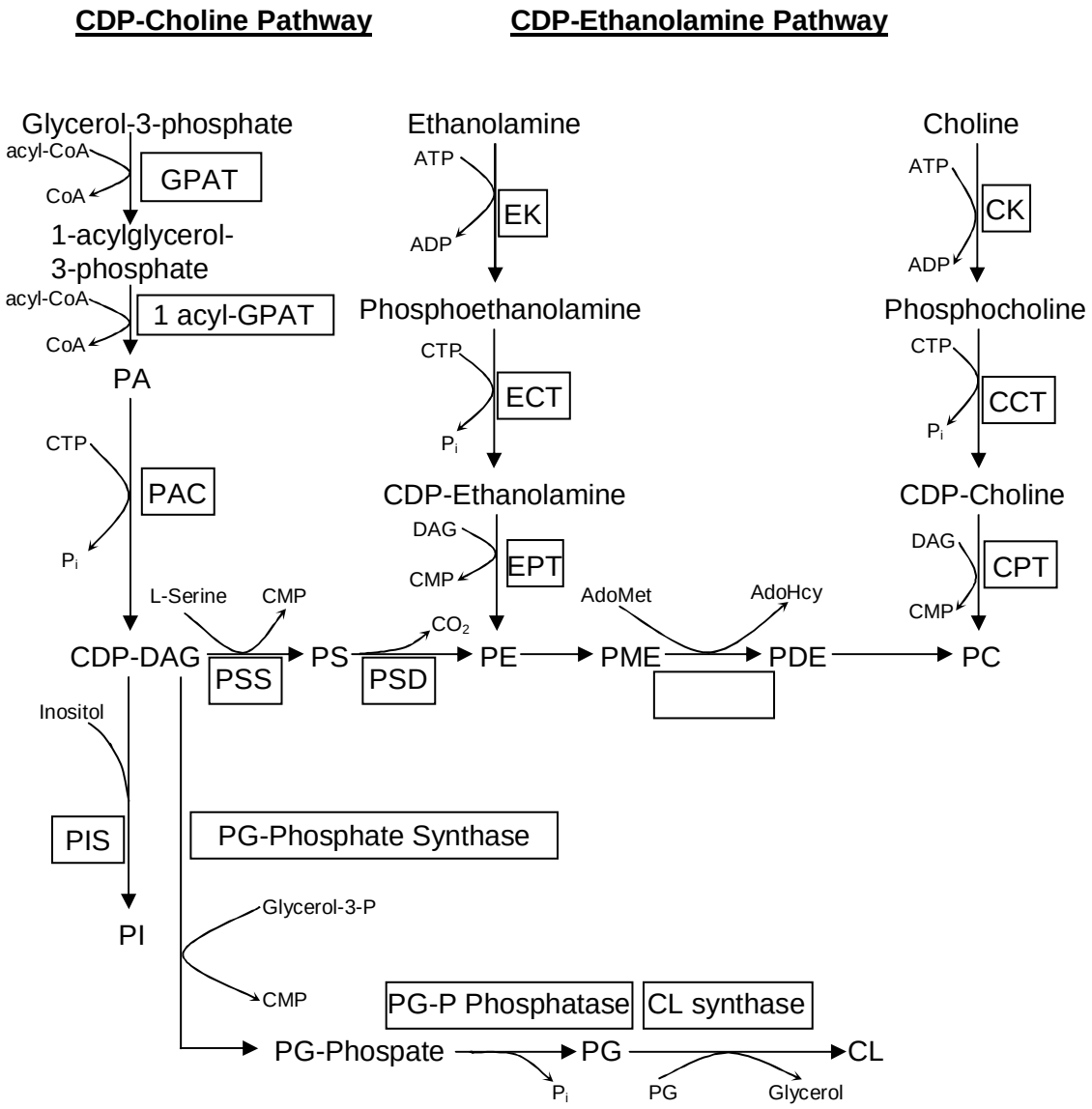


Figure 1.3: Pathways for the biosynthesis of major eukaryotic phospholipids (Kent & Carman, 1999; Zubay, 1998). Enzyme involved in individual steps are boxed. CK, choline kinase; EK, ethanolamine kinase; CCT, choline phosphate cytidyltransferase; ECT, ethanolamine phosphate cytidyltransferase; CPT, choline phosphotransferase; EPT, ethanolamine phosphotransferase; GPAT, glycerol-3-phosphate acyltransferase; 1 acyl-GPAT, acyltransferase; PAC, phosphatidate cytidyltransferase; PSS, phosphatidylserine synthase; PSD, phosphatidylserine decarboxylase; PEMT, phosphatidylethanolamine N-methyltransferase; PIS, phosphatidylinositol synthase; PG-P synthase, phosphatidylglycerol phosphate synthase; PG-P phosphatase, phosphatidylglycerol phosphate phosphatase; CL synthase, cardiolipin or diphosphatidylglycerol synthase; PA, phosphatidic acid; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PME, phosphatidylmonoethanolamine ; PDE, phosphatidyldiethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; CL, cardiolipin (diphosphatidylglycerol); DAG, diacylglycerol; AdoMet, S-adenosylmethionine; AdoHcy, adenosylhomocystein. Conversion of PC or PE to PS by base exchange reaction

1.2.2 Synthesis of phosphatidylcholine

Generally, there are two pathways for PC biosynthesis (Figure 1.3), namely the CDP-choline pathway and the PE methylation pathway. The PE methylation pathway predominates in yeast (Kent, 1995) and mammalian liver cells, whereas the CDP-choline pathway is predominant in other mammalian cells (DeLong *et al*., 1999). The CDP-choline pathway in yeast becomes more important when enzymes of the CDP-diacylglycerol pathway are defective or repressed (Carman & Henry, 1989). The yeast CDP-choline pathway also acts as salvage pathway for choline that resulted from turnover of PC synthesized by CDP-diacylglycerol pathway (McMaster & Bell, 1994a). In fact, mutants defective in CDP-choline pathway enzymes, such as choline kinase, excrete choline into the growth medium as a result of PC turnover and their inability to reutilize choline for PC synthesis by the CDP-choline pathway (Patton-Vogt *et al*., 1997). PC synthesized by CDP-choline and CDP-diacylglycerol may have distinct as well as overlapping functions in cell physiology (Carman & Kersting, 2004).

In prokaryotes, PC biosynthesis was thought to occur only by PE methylation, however, a second pathway for PC biosynthesis that is distinct from CDP-choline pathway and seems to be exclusive to bacteria, known as the phosphatidylcholine synthase (PCS) pathway, has recently been discovered (Sohlenkamp *et al*., 2000). In this pathway, choline is condensed directly with CDP-diacylglycerol to form PC and CMP in a reaction catalysed by PCS (Martinez-Morales *et al*., 2003).

1.2.2.1 CDP-choline pathway

The CDP-choline pathway (Figure 1.3) was first described by Eugene Kennedy who discovered that CTP, rather than ATP, was the activating nucleotide for this biosynthetic pathway (Kennedy & Weiss, 1956). The committed step in the synthesis of PC is catalyzed by choline kinase that phosphorylates choline to form phosphocholine with ATP as the phosphoryl donor. CTP:phosphocholine cytidyltransferase

subsequently converts phosphocholine into CDP-choline with CTP. The final step in the CDP-choline pathway is catalyzed by choline phosphotransferase that catalyzes the formation of PC from CDP-choline and diacylglycerol (Carman & Henry, 1999; Kent, 1995; Vance & Vance, 2004). Under normal metabolic conditions, the CDP-choline pathway is regulated by the second enzyme in the pathway, CTP:phosphocholine cytidyltransferase (Vance & Vance, 2004). However, the first enzyme in this pathway, choline kinase can also be regulatory at choline concentration higher than 100 µM (McMaster & Bell, 1994b).

Since choline is not synthesized *de novo* in animal cells, this important PC precursor has to come from the diet, or must be added to cell culture medium. Two classes of transporter have been proposed for choline transport into cells (Ishidate, 1997). The first type of transporter is a high affinity sodium-dependent transporter (Yamamura & Snyder, 1972), whereas the second type is a low affinity sodium-independent transporter (Ferguson *et al*., 1991).

1.2.2.2 Phosphatidylethanolamine methylation pathway

The phospholipid N-methyltransferases catalyze the three step AdoMet-dependent methylation of PE to PC as shown in Figure 1.3 (Carman & Henry, 1999). In yeast, the first methylation reaction is catalyzed by the PE methyltransferase (Kodaki & Yamashita, 1987), and the last two methylation reactions are catalyzed by phospholipid methyltransferase (Gaynor & Carman, 1990). Mammalian PE methyltransferase was first purified from rat liver and, unlike in yeast, this enzyme catalyzes all three methylation reactions of the conversion from PE to PC (Ridgway & Vance, 1988).

1.2.3 Synthesis of phosphatidylethanolamine

The two major biosynthetic pathways of PE are the CDP-ethanolamine pathway as originally described by Kennedy and Weis (1956) and the PS decarboxylation pathway

(Vance & Vance, 2004). A small amount of PE can also be synthesized by base-exchange reaction as catalyzed by PS synthase-2 (Sundler *et al*., 1974). The importance of the CDP-ethanolamine pathway and of the PS decarboxylation pathway are cell type-dependent. Typically, BHK (Voelker, 1984) and CHO cell lines (Miller & Kent, 1986) have more than 80% of their PE synthesized through the PS decarboxylation pathway even when the cells are supplied with sufficient ethanolamine to support PE synthesis via the CDP-ethanolamine pathway. In contrast, the CDP-choline pathway has been reported to be the prominent PE synthesis pathway in rat liver and hepatocytes as well as hamster heart (Vance & Vance, 2004).

1.2.3.1 CDP-ethanolamine pathway

The committed step in the synthesis of PE via the CDP-ethanolamine pathway (Figure 1.3) is catalyzed by ethanolamine kinase. This enzyme phosphorylates ethanolamine to yield phosphoethanolamine, using ATP as phosphoryl donor. The second step is catalyzed by CTP:phosphoethanolamine cytidyltransferase which catalyzes the formation of CDP-ethanolamine from CTP and phosphoethanolamine. As in the parallel CDP-choline pathway, this step is believed to be the rate limiting step in the CDP-ethanolamine pathway. The last step in the CDP-ethanolamine pathway is catalyzed by ethanolamine phosphotransferase that catalyzes the formation of PE from CDP-ethanolamine and diacylglycerol (Kennedy & Weiss, 1956; Vance & Vance, 2004).

1.2.3.2 Phosphatidylserine decarboxylation pathway

The conversion of PS to PE is catalyzed by PS decarboxylase (Figure 1.3). There are two genes encoding PS decarboxylase in *S. cerevisiae*, PSD1 and PSD2 (Carman & Kersting, 2004). The PSD1-encoded enzyme is responsible for more than 90% of the PS decarboxylase activity in yeast cells (Carman & Kersting, 2004). In contrast, all PS decarboxylase activity in mammalian cells appears to result from the product of a single gene (Kuge *et al*., 1991). Production of PE from decarboxylation of PS is limited

by the transfer of newly synthesized PS (mostly in ER) to the location of PS decarboxylase in the mitochondrial inner membrane (Voelker, 1989).

1.2.4 Synthesis of Phosphatidylserine

Phosphatidylserine (PS) is synthesized by phosphatidyl transfer from either CDP-diacylglycerol (in yeast and prokaryotes) as shown in Figure 1.3, or PC/PE in mammalian cells (Kent, 1995; Vance & Vance, 2004). Phosphatidyl transfer from PC or PE is also known as base-exchange reaction (Kent, 1995). PS synthase catalyzes the transfer of the phosphatidyl moiety from CDP-diacylglycerol to serine. CDP-diacylglycerol is synthesized from CTP and PA by CDP-diacylglycerol synthase (Vance & Vance, 2004). In the base exchange reaction, the head group of preexisting phospholipids (PC and PE) is replaced by L-serine (Vance, 2003). The reaction is catalyzed by two distinct calcium-dependent PS synthases: PS synthase 1 exchanges serine for the choline head group of PC, whereas PS synthase 2 uses PE (Vance & Vance, 2004). In fact, there may be more than two base exchange enzymes in mammalian cells, with enzymes in different tissues and subcellular locations exhibiting distinct substrate specificities and abilities to be stimulated by agonists (Siddiqui & Exton, 1992).

1.3 Choline kinase (CK) and ethanolamine kinase (EK)

CK (ATP:choline phosphotransferase, EC 2.7.1.32) and EK (ATP:ethanolamine phosphotransferase, EC 2.7.1.82) catalyze the phosphorylation of choline/ethanolamine by ATP in the prescence of Mg^{2+}, yielding phosphocholine/phosphoethanolamine and ADP (Ishidate, 1997). These enzyme steps commit choline/ethanolamine to the so-called Kennedy pathway (CDP-choline or CDP-ethanolamine pathway) for biosynthesis of PC and PE (Figure 1.3).

It has been a controversial issue as to whether the same enzyme catalyzes the phosphorylation of choline and ethanolamine since highly purified CK from rat kidney (Ishidate *et al*., 1984), liver (Porter & Kent, 1990) and brain (Uchida & Yamashita, 1990) all showed EK activity, though with considerable preference for choline. The nomenclature of choline kinase therefore became choline/ethanolamine kinase (CK/EK). In *S. cerevisiae*, there are two enzymes that are able to phosphorylate choline and ethanolamine, and these enzymes are annotated based on their preferred substrate specities (Lykidis *et al*., 2001). The choline kinase (CKI) has 3.6 fold higher specific activity with choline compared to ethanolamine, whereas the ethanolamine kinase (EKI) has 2 fold higher specific activity with ethanolamine compared to choline (Kim *et al*., 1999).

On the contrary, there have been some earlier reports which suggested the existence of separate EK in mammalian species (Brophy *et al*., 1977; Draus *et al*., 1990; Upreti *et al*., 1976; Weinhold & Rethy, 1974), and CK/EK activities have also been shown to be separable in several lower eukaryotes and plants (Ishidate, 1997; Wharfe & Harwood, 1979). The discovery of an ethanolamine-specific kinase in *Drosophila* (Pavlidis *et al*., 1994) has also led to the speculation that EK, which is essentially devoid of CK activity, may also exist in mammalian species. Only recently, a human ethanolamine specific kinase cDNA was cloned by Lykidis *et al*. (2001). In addition, the existence of monomethylethanolamine kinase and dimethylethanolamine kinase, which are different from either CK or EK, has been reported in rat brain (Cao & Kanfer, 1991).

CK/EKs are ubiquitously distributed in eukaryotes from yeast to mammals and plants (Yamashita & Hosaka 1997). CK/EKs are generally accepted as cytosolic and can be handled as soluble enzymes by conventional purification procedures (Yamashita & Hosaka, 1997). Particularly, mammalian CK, besides its conventional role in PC synthesis, has also been implicated in cancer pathogenesis, and its inhibition has been

proposed as a promising anticancer strategy (Ramirez de Molina, 2002a). In addition, increased CK activity in response to treatment of rats with aromatic hydrocarbons (Ishidate *et al*., 1982), or treatment of cultured cells with growth stimulants such as serum, epidermal growth factors, or insulin (Uchida, 1996; Warden & Friedkin, 1985), has also been reported.

1.3.1 Purification and substrate specificity of choline/ethanolamine kinase

Choline kinase was first purified by Wittenberg & Kornberg (1953) with a 25-fold enrichment of enzyme activity from brewer's yeast autolysate. Until another attempt to purify the yeast choline kinase by Brostrom & Browning (1973), the enzyme was used for practical purposes such as for the determination of acetylcholine in biological materials (McCaman *et al*., 1971). Partially purified yeast choline kinase was shown to mediate the phosphorylation of not only choline, but also N,N-dimethylethanolamine, N-monomethylethanolamine, and ethanolamine (Wittenberg & Kornberg, 1953). The ability of yeast choline kinase to phosphorylate ethanolamine besides choline has subsequently been confirmed by gene disruption studies showing functional complementation (Hosaka *et al*. 1989) and characterization of purified recombinantly produced choline kinase (Kim *et al*., 1998).

Different characteristics of choline kinases from various mammalian tissues strongly indicated that choline kinase does exist in several isoforms (Ishidate, 1997). This was subsequently confirmed by cDNA cloning of three different rat choline kinase isoforms (Aoyama *et al*., 1998b; Uchida, 1994b; Uchida & Yamashita, 1992b). It is now generally accepted that mammalian cells have at least three choline kinase isoforms (termed α1, α2, and β) and the active enzyme consists of either their homo- or hetero-dimeric (or oligomeric) species (Aoyama *et al*., 2004). Table 1.3 shows the characteristics of CKs obtained with enzymes that were highly purified from mammalian sources or produced recombinantly. Currently, highly purified ethanolamine-specific kinases are still

Table 1.3: Characteristic parameters of CK obtained with highly purified or recombinant enzyme preparations. All of these highly purified CKs also showed EK activities. S.A, specific activity. Table is adapted from Aoyama *et al*. (2004).

Source	SDS-PAGE (kDa)	Native form	S.A (mmol/min/mg)	K_m Choline (µM)	K_m ATP (mM)	Putative isoform
Rat kidney[a,b]	42	dimer	3.3	100	1.5	CKβ
Rat liver[c]	47	tetramer	143	13	0.04	CKα2
Rat brain[d]	44	dimer	40	14	1,0	CKα1
S. cerevisiae[e] (recombinant)	73	dimer	128	270	0.09	CKI
C. elegans[f] (recombinant)	48 40	dimer (oligomer)	43 2.4	1.6 mM 13 mM	2.4 0.72	CKA-2 CKB-2

[a] Ishidate *et al*. (1984)
[b] Ishidate *et al*. (1985)
[c] Porter & Kent (1990)
[d] Uchida & Yamashita (1990)
[e] Kim *et al*. (1998)
[f] Gee & Kent (2003)

unavailable; only partial purification of EKs from rat liver (Uchida, 1997; Weinhold & Rethy, 1974) and recombinantly produced GST-tagged *Drosophila* EK (Uchida, 1997) have been reported.

1.3.2 Molecular cloning of eukaryotic choline kinase

Table 1.4 shows the cDNA cloning of CK and EKs from various organisms together with their nomenclatures and properties. The first CK structural gene was isolated from *S. cerevisiae* by means of genetic complementation, using a mutant defective in CK activity (Hosaka *et al*.,1989). This CK was termed CKI; no intron was present in its protein coding sequence. The clone had an open reading frame of 582 amino acids with calculated molecular size of 66,316 Da. The cloned gene could be expressed in *E. coli* and the resulting transformed cells displayed significant CK activity. The transformed bacteria also displayed EK activity, indicating that both activities could be encoded by the same gene in yeast (Hosaka *et al*., 1989). Subsequent expression, purification and characterization of yeast CK expressed in Sf-9 insect cells confirmed its CK activity and showed that ethanolamine was a poor substrate compared to choline (Kim *et al*., 1998).

In *S. cerevisiae*, Sec14p is a PI/PC transfer protein that is essential for cell viability and vesicle budding from the Golgi complex (Fang *et al*., 1998). It has been proposed that the function of Sec14p is to down-regulate the synthesis of PC via the CDP-choline pathway (Fang *et al*., 1998). It appears that too much PC synthesized via the CDP-choline pathway is detrimental to the secretory process (Kent & Carman, 1999). Interestingly, mutations in the CKI gene can suppress (bypass) the lethal phenotype of *sec14* mutants (Cleves *et al*., 1991) by causing a block in the CDP-choline pathway, which in turn excludes the need for Sec14p function (Cleves *et al*., 1991; Fang *et al*., 1998).

Table 1.4: Cloned CK and EK from various organisms. Ethanolamine specific EK from human and *D. melanogaster* are marked by *.

Source	Nomenclature	Putative number of amino acid	Calculated molecular weight (kDa)
Rat liver	CKR1(CKα1)[a]	435	50
	CKR2(CKα2)[a]	453	52
Rat kidney	CKβ[b]	394	45
Mouse embryo	CKα1[c]	435	50
	CKα2[c]	453	52
	CKβ[c]	394	45
Human glioblastoma	hCKα2[d]	456	52
Human liver	*EKI-1[e]	452	50
	EKI-2α[e]	477	-
	EKI-2β[e]	394	-
S. cerevisiae	CKI[f]	582	66
	EKI[g]	534	62
C. elegans	CKA-2[h]	429	49
	CKB-2[h]	369	42
Drosophila melanogaster	*dEK (eas)[i]	517	59
Soybean	GmCK1[j]	359	41
	GmCK2[j]	362	42
	GmCK3[j]	>497	>56
Pea	CK[k]	343	40

[a] Uchida & Yamashita (1992b)
[b] Uchida (1994b)
[c] Aoyama *et al*. (1998a)
[d] Hosaka *et al*. (1992)
[e] Lykidis *et al*. (2001)
[f] Hosaka *et al*. (1989)
[g] Kim *et al*. (1999)
[h] Gee & Kent (2003)
[i] Monks *et al*. (1996)
[j] Al-Malki *et al*. (2000)

Cloning of the first mammalian CK was reported by Uchida & Yamashita (1992b). By screening a rat liver cDNA expression library constructed in *λgt11* with antibodies raised against highly purified rat liver choline kinase, an open reading frame encoding 435 amino acids with a calculated molecular size of 49,743 Da was isolated and confirmed to be the structural gene of choline kinase termed CKR1 (Uchida & Yamashita, 1992b). This enzyme was found to mediate the phosphorylation of both choline and ethanolamine. Subsequently, the cDNA of a CKR1-related isoform, termed CKR2, was cloned by Uchida (1994b) from the same cDNA library; it contained an extra 54 bases internal sequence probably arising through alternative splicing. Thus, CKR2 is predicted to encode a protein of 453 amino acids with a molecular weight of 51,824 Da. Southern blot analysis indicated a single copy of the CKR gene. The genomic DNA containing the first exon and its flanking regions was isolated and partially characterized (Uchida, 1994b). The CKR gene showed not only characteristics of a typical housekeeping gene but also of a gene regulated through a variety of putative cis-acting elements. The most interesting point could be an involvement of both the putative xenobiotic and antioxidant responsive elements in the 5' flanking region of the CKR gene (Uchida, 1994b).

More recently, by means of peptide sequence information of a 42 kDa (estimated by SDS-PAGE) choline kinase, a cDNA clone encoding 394 amino acids with a calculated molecular size of 45,100 kDa has been cloned from a rat kidney cDNA library. Significant CK and EK activities appeared in cell lysate of *E. coli* transfected with plasmid carrying this ORF (Aoyama *et al*., 1998b). Amino acid sequence comparison of this CK with previously cloned CKR1 and CKR2 showed only 57 to 59% identity, indicating that the cloned cDNA must be a product of a gene distinct from that for CKR (Aoyama *et al*., 1998b). Altogether, three isoforms of CKs have been cloned from rat, and the nomenclatures of CKR1, CKR2 and 42 kDa CK/EK were renamed by Aoyama *et al*. (2000) as CK/EKα1, CK/EKα2 and CK/EKβ, respectively. CK/EKα and CK/EKβ

are encoded by two distinct genes, and all three choline kinase isoforms exhibit significant ethanolamine kinase activity.

Human CK cDNA was cloned from a glioblastoma cDNA expression library by complementation of the same yeast *cki* mutant used in cloning of *S. cerevisiae* CK (Hosaka *et al*., 1992). The deduced sequence of the human enzyme comprised 456 amino acids with a calculated molecular weight of 52,065 Da. The cDNA was confirmed to encode CK when an *E. coli* expression plasmid yielded highly increased CK activity in bacterial cell lysate, and when cloned into an *S. cerevisiae* vector it also managed to rescue the yeast *cki* mutant (Hosaka *et al*., 1992). Based on sequence identities, the human CK was identified as homolog of the rat CK/EKα2 isoform, hence, it was termed hCKα2 (Aoyama *et al*., 2000; Aoyama *et al*., 2004). The human homolog of rat CK/EKβ was identified by Yamazaki *et al*. (1997) while characterizing the gene structure of human muscle type carnitine palmitoyltransferase I (CPTI-M). Human CKβ (hCKβ) gene is located only about 300 bp upstream from exon 1A of the human CPTI-M gene. The predicted cDNA sequence of hCKβ was subsequently reported by Aoyama *et al*. (2000). However, hCKβ has never been characterized and its choline kinase activity has yet to be confirmed.

Mouse putative choline kinases, CK/EKα1 and CK/EKβ were cloned by Aoyama *et al*. (1998a) from a mouse embryo cDNA library. Amino acid sequences of both mouse choline kinase isoforms share high sequence identity with the respective rat CK/EKα1 (96.0%) and CK/EKβ (92.4%). However, characterizations of both isoforms have never been reported.

Recently, cloning and characterizations of CKs from *Caenorhabditis elegans* have been reported by Gee & Kent (2003). They cloned five and expressed four out of seven

genes that were found in the *C. elegans* genome and that are likely to encode choline and/or ethanolamine kinases. The proteins were expressed in Sf-9 insect cell and showed CK activity. Those that resemble the mammalian CKs were the most active ones (Gee & Kent, 2003). The most active isoform, CKA-2, was purified to near homogeneity; it showed similarly high catalytic activity compared to CKs from rat liver and yeast (Gee & Kent, 2003). Another purified and characterized isoform is CKB-2; it has much lower activity compared to the CKA-2 isoform (Gee & Kent, 2003). CKA-2 is also the first CK whose crystal structure was solved (Peisach *et al*., 2003).

Molecular cloning of a plant CK was first reported by Monks *et al*. (1996) for soybean (*Glycine max*). Two distinct full length cDNAs encoding two putative CKs, designated GmCK1 and GmCK2, were isolated from a soybean cDNA library. Heterologous expression of GmCK1 in yeast (*S. cerevisiae*) and GmCK2 in yeast and *E. coli* confirmed that both encode active CKs (Monks *et al*., 1996). However, unlike the CKs from animals and yeast, soybean choline kinase did not show any significant EK activity when the cell extracts from yeast and *E. coli* expressing GmCK1 and GmCK2 were assayed for ethanolamine phosphorylating activity (Monks *et al*., 1996).

Full length cDNA from pea that encodes a putative CK protein of 343 amino acids (with a calculated molecular weight of 39,785 Da) has been cloned and expressed in *E. coli* (Al-Malki *et al*., 2000). Induced expression of the protein encoded by the pea cDNA in *E. coli* produced significant amount of CK activity in the *E. coli* cell extract (Al-Malki *et al*., 2000).

As summarized in Table 1.3, only recombinantly produced *S. cerevisiae* and *C. elegans* CKs have been purified to homogeneity. Both were expressed in Sf-9 insect cells using the baculovirus system (Gee & Kent, 2003; Kim *et al*., 1998). Surprisingly little attention has been given to molecular cloning of different isoforms of human CK.